ELSEVIER

FEMS Microbiology Letters 135 (1996) 85–92

FEMS MICROBIOLOGY LETTERS

Osa protein encoded by plasmid pSa is located at the inner membrane but does not inhibit membrane association of VirB and VirD virulence proteins in *Agrobacterium tumefaciens*

Chao-Ying Chen [1], Clarence I. Kado *

Davis Crown Gall Group, Department of Plant Pathology, University of California, Davis, CA 95616, USA

Received 14 September 1995; revised 7 October 1995; accepted 17 October 1995

Abstract

The *osa* gene of IncW plasmid pSa encodes a 21-kDa protein that completely abolishes the oncogenic activity encoded by virulence genes in *Agrobacterium tumefaciens*. *osa* is the last gene of a four-gene operon in pSa, the expression of which appears to be highly regulated since the Osa protein is absent when either pSa or the *osa* operon is present in the *Agrobacterium* cell. When the *osa* gene alone or together with upstream genes within the operon are expressed under the control of a constitutive promoter, Osa protein is produced, enabling us to determine its subcellular location. Immunoblot analyses located Osa protein at the inner membrane of both *A. tumefaciens* and *Escherichia coli*. Because Osa inhibits oncogenicity of *A. tumefaciens*, and because alterations of the products of the *virB* and *virD* genes affect oncogenicity, studies were conducted to determine if there are changes in their specific association with the membranes in the presence Osa. Immunoblot analyses of VirB2, VirB3, VirB4, VirB9, and VirD4 in the presence and absence of Osa revealed no differences between the two treatments in these Vir protein associations with the membranes. These results indicate that both *virB* and *virD* gene products are produced in the presence of Osa; that they appear unaffected in their association with the membranes; and that Osa is associated with the inner membrane, where VirB2, VirB4, and VirD4 proteins are also located.

Keywords: *Agrobacterium tumefaciens*; Oncogenicity; Crown gall tumors; IncW plasmid; pSa plasmid; Ti plasmid; RP1 plasmid; Vir proteins; Fertility inhibition

1. Introduction

The oncogenicity of *Agrobacterium tumefaciens* is dependent on the presence of the Ti plasmid, which contains virulence (*vir*) genes necessary for the delivery of a specific 25-kb sector (the T-DNA containing oncogenes) of this plasmid into plant cells, culminating in the incorporation of this DNA into the plant genome. Mutations of *vir* genes such as *virB* and *virD* genes required for the processing and transfer of the T-DNA results in the loss of oncogenicity. Mutations of chromosomal genes such as *chv* genes involved in virulence also appreciably affect oncogenicity. An ancillary phenomenon of oncogenic suppression has been observed by the transfer of the IncW plasmid pSa into *A. tumefaciens* [1]. The oncogenic inhibition by pSa is absolute, indicating that some step in the T-DNA processing or transfer is completely blocked in the presence of pSa. The genetic element on pSa conferring this

* Corresponding author.

[1] Present address: Department of Plant Pathology and Entomology, National Taiwan University, Taipei, Taiwan 106.

SSDI 0378-1097(95)00434-3

activity rests in a single gene, *osa* (for *o*ncogenic *s*uppressive *a*ctivity) [2]. This gene is part of the *osa* operon containing at least four open reading frames (orfs) [3]. The functions of *orf1* and *orf3* are unknown, while *orf2* encodes a nuclease [4], and *orf4* encodes the 21-kDa Osa protein [2]. Amino acid sequence analysis of Osa has revealed an interesting homology to proteins involved in fertility inhibition of pSa by IncP plasmid RP1 [3], suggesting that Osa might also play a role in fertility inhibition such as affecting delivery of the T-DNA by a conjugative mechanism [3]. Credence of this hypothesis comes from the fact that the *virB* genes are directly involved in T-DNA transfer, and are homologs of genes involved in the synthesis and assembly of a conjugative pilus [5,6]. Nonetheless, the mechanism by which Osa blocks T-DNA delivery is not precisely understood; its activity is not associated with either the inhibition of transcription of *vir* genes, or preventing the production of T-DNA intermediates [7]. Useful information can be obtained from amino acid sequence data; however, examination of the amino acid sequence of Osa has not revealed any homologies to known proteins. The sequence showed no potential signal sequences, but a region rich in hydrophobic amino acid residues near its carboxyl terminus is present, suggesting that Osa might potentially associate with the bacterial membrane [3]. Because a number of possibilities as to the function of Osa have been ruled out, the subcellular location of Osa would provide an important clue leading to its function.

In this communication, we show that Osa is associated with the inner membrane of *A. tumefaciens*. Since many of the VirB proteins are associated with the inner membrane [9–14], one notion has been that there may be competitive inhibition between VirB and Osa proteins. However, such competition appears not to occur.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and plasmids

To express Osa protein, *Escherichia coli* BL21(DE3) containing a chromosomal T7 RNA polymerase gene under the control of a *lacUV5* promoter [15] was used as a host for plasmid pET-3a [16] containing the *osa* gene under the control of a T7 promoter. The cells were grown at 37°C in medium 2YT [17] containing 100 μg ml^{-1} ampicillin. *E. coli* DH5α containing pUCD3960 [3] was grown in a similar fashion and used as a host for Osa production. *A. tumefaciens* LBA4301 (pTiC58Tra^c) was grown in AB induction medium [18] at 28°C. LBA4301 cells were exposed to 200 μM acetosyringone (= 3′,5′-dimethoxy-4′-hydroxy-acetophenone) at mid-exponential phase and were allowed to grow for an additional 14 h before they were collected by centrifugation (5000 × *g*, 4°C, 10 min).

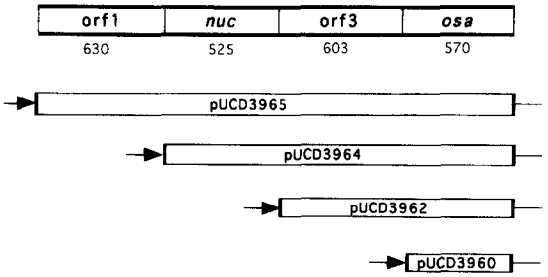

Fig. 1. Organization of the *osa* operon and its deletion derivatives. Each deletion derivative contains the indicated orfs or genes, of which the upstream most gene is fused to the constitutive kanamycin/spectinomycin resistance operon promoter (arrow). The length in base pairs is indicated below each orf.

The *osa* gene was cloned into pET-3a that had been cleaved by double digestion with restriction enzymes *Bam*HI and *Nde*I. The *osa* gene with flanking *Bam*HI and *Nde*I cleavage sites generated by polymerase chain reaction amplification was inserted in the respective cloning site of pET-3a. The resulting plasmid designated pUCD3985 was transferred to *E. coli* BL21(DE3) by electroporation. In addition, as shown in Fig. 1, progressive deletion derivatives of each *orf* of the *osa* operon were cloned in the *Kpn*I restriction site within pUCD105 described previously [3], and resulted in recombinant plasmids pUCD3965, pUCD3964, pUCD3962, and pUCD3960, each under the control of a promoter of the kanamycin/spectinomycin operon described previously [8]. Each clone was transformed into *E. coli* DH5α. We also used pUCD1311, which contained a 3.1-kb DNA sector of pSa containing the entire *osa* operon [2]. pUCD3940-30 is a derivative of

pUCD1311 containing a TnCAT insertion 2025 bp upstream of the 5′-end of *osa* [3].

2.2. Protein purification, antibody production, and immunoblotting

Osa protein was produced in *E. coli* BL21(DE3) cells containing the *osa* gene cloned in pET-3a and designated as pUCD1318. At mid-exponential phase, the cells were exposed to 0.4 mM IPTG (= isopropyl-β-D-thiogalactopyranoside) and incubated for 2 h. They were then lysed by sonication at maximum setting with five 20-s pulses using an ultrasonicator (Heat Systems-Ultrasonics, Inc., Formingdale, NY). The proteins were fractionated by SDS-PAGE [19], transferred to nitrocellulose membrane (0.45 mm, BA85, Schleicher and Schuell, Inc.), and visualized by staining with Ponceau S. The banded protein was excised, sliced into small pieces and ground into a powder in liquid nitrogen with a mortar and pestle. The powder was resuspended in normal saline and used as the immunogen in the presence of complete adjuvant. Rabbit antiserum was raised by Antibodies, Inc., Davis, CA. Non-specific cross-reacting antibodies were removed by immunocross-absorption with clairfied whole-cell lysates of *E. coli* Bl21(DE3) containing pET-3a, and with that of *A. tumefaciens* LBA4301 (pTiC58Tra^c) containing cloning vector pUCD105 [20].

Proteins from total cell lysates were fractioned into subcellular components by SDS-PAGE and transferred by electroblotting to nitrocellulose membranes. Proteins on the membrane were reacted with specific Osa antibodies followed by goat anti-rabbit horse raddish peroxidase-conjugated secondary antibodies commercially supplied in the ECL system (Amersham, Arlington Heights, IL). Polyclonal rabbit antibodies against *A. tumefaciens* Vir proteins, VirB2, VirB3, VirB4, VirB9, and VirD4, were raised at Antibodies, Inc., Davis, CA.

2.3. Fractionation of subcellular components

Periplasmic, cytoplasmic, inner and outer membrane components were fractionated by differential centrifugation [21]. All steps were performed at 4°C. *A. tumefaciens* with or without acetosyringone was suspended in 5 ml lysis buffer (25 mM HEPES, pH 7.6, 20% sucrose, 2 mM EDTA, 0.2 mg ml^{-1} lysozyme) for 30–60 min. The lysate was clarified by centrifugation (7000 × *g*, 15 min). The resulting pellet was resuspended in 5 ml HEPES buffer (25 mM, pH 7.6) and sonicated three times for 20 s each at full output. Cellular debris was removed by centrifugation (7000 × *g*, 15 min) and KCl was added to the supernatant to a final concentration of 0.2M. The suspension was centrifuged at 110000 × *g* for 1 h to yield the cytoplasmic fraction. The inner and outer membranes were obtained from cells collected by centrifugation of 250 ml of culture at mid-exponential phase. The cells were resuspended in 10 ml of a solution containing 25 mM HEPES, pH 7.6, 20% sucrose, 0.2M KCl, 0.2 mM dithiothreitol, 0.2 mg ml^{-1} desoxyribonuclease, and 0.2 mg ml^{-1} ribonuclease A, and passed through a French pressure cell (Amicon, Beverly, MA) three times at 16000 psi. Lysozyme was then added to the cracked cell suspension to a final concentration of 1 mg ml^{-1}. The mixture was incubated for 30–60 min and then centrifuged (7000 × *g* for 10 min) to remove cellular debris. The supernatant containing the cell envelopes was centrifuged at 110000 × *g* for 1 h and the resulting pellet was resuspended in 1 ml solution containing 5 mM EDTA, pH 7.5, 0.2 mM dithiothreitol and 20% sucrose. This suspension was layered with a discontinuous density gradient consisting of 1 ml of 70% sucrose on top of which 2.9 ml of 53% sucrose was layered, both in 5 mM EDTA, pH 7.5. The gradient was centrifuged at 100000 × *g* for 24 h to separate the inner and outer membranes. Fractions (0.5 ml) were collected from the bottom of the gradient and each fraction was assayed for protein concentration using a protein assay reagent and analysed by the Softmax program (BioRad, Hercules, CA). NADH-oxidase activity, naturally associated with the inner membrane and serving as a marker for this subcellular fraction, was analysed as described previously [11].

3. Results

3.1. Isolation and purification of Osa

Osa protein was produced in *E. coli* DH5α containing pUCD3960 and the whole cell lysate was

analysed by SDS-PAGE. Densitometry of the fractionated proteins showed that Osa represented approximately 0.2% of the total cellular protein in exponential phase cells, which therefore provided a minimal source of the protein (Fig. 2). On the other hand, Osa produced in *E. coli* BL21(DE3) cells containing pUCD3985 represented approximately 5% of the total cellular protein. Excessive production of Osa stimulated by subjecting the cells to IPTG was lethal to the cells. Thus, even in the absence of induction with IPTG, there was sufficient expression of the *osa* gene that resulted in Osa production, which was amenable for purification. Cell lysates of the latter host were therefore routinely used to obtain Osa which conveniently fractionated into the insoluble fraction (Fig. 2). Proteins in this fraction were fractionated electrophoretically by SDS-PAGE and the separated proteins were transferred by electroblotting to a nitrocellulose membrane. The membrane portion, containing the Osa protein, visualized by staining with Ponceau S, was used to raise polyclonal antibody purified further by cross absorption as described in Materials and methods. The specificity of the antibody was demonstrated by Western blotting whole-cell lysates prepared from *E. coli* BL21(DE3) with and without the *osa* gene cloned in its vector plasmid (Fig. 2). Only those cells bearing *osa* displayed a protein band reactive to the antibody.

3.2. Osa protein production in A. tumefaciens is regulated

Whole-cell lysates prepared from *A. tumefaciens* LBA4301(pTiC58Tra^c) harboring either pSa or pUCD1311 were analysed by SDS-PAGE and by immunoblotting with Osa antibody. The Osa protein was barely detectable by staining with Coomassie brilliant blue and by immunoblotting, suggesting that the expression of *osa* is regulated in this organism (Fig. 2). As expected, the interuption of transcription of the *osa* operon by the insertion of TnCAT upstream of *osa* failed to produce the Osa protein.

Since *osa* is part of an operon containing three additional genes upstream of *osa* [3], clones bearing a systematic deletion of each of these genes with a constitutive promoter driving the remaining genes downstream of the deletion (Fig. 1) were analysed in the above *A. tumefaciens* test strain. Cells contain-

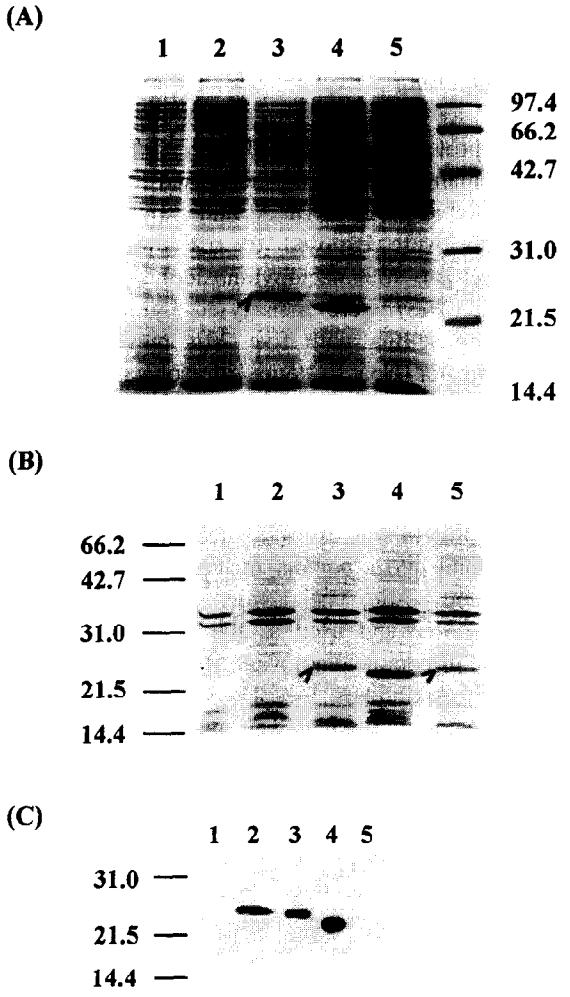

Fig. 2. SDS-PAGE analyses of soluble and insoluble proteins prepared from *E. coli* DH5α cells containing one of the following plasmids: (**A**) soluble fraction: pUCD105 (lane 1); pUCD3960 (lane 2); pUCD3985 (lane 3); pUCD1318 (lane 4); and pET-3a (lane 5). The protein band (arrow) in lane 3 is a fusion protein between Osa and the first 12 amino terminal amino acid residues of T7φ10 gene of pET-3a, resulting in a protein larger than the expected size of Osa (lane 4). (**B**) Insoluble fraction: pUCD105 (lane 1); pUCD3960 (lane 2); pUCD3985 (lanes 3 and 5); pUCD1318 (lane 4). The same fusion protein described above is indicated by the arrow in lanes 3 and 5. The protein bands resolved in 15% acrylamide gels were stained with Coomassie brilliant blue. (**C**) Western blot of Osa as the fusion protein (described in **A**) shown in lanes 2 and 3, and Osa protein (lane 4). Lanes 1 and 5 contain proteins derived from cells with the respective vectors pUCD105 and pET-3a. Specific Osa antibody was used and detected by goat anti-rabbit antibody conjugated with horse radish peroxidase.

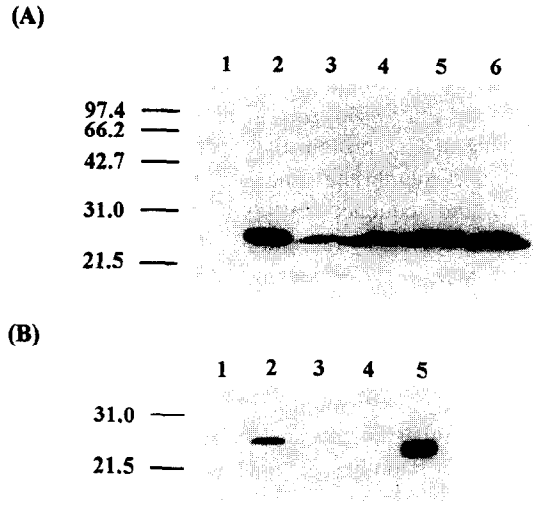

Fig. 3. Western blot analysis of *A. tumefaciens* LBA4301(pTiC58Tra^c) containing the following plasmids: (**A**) whole cell lysate: pUCD105 (lane 1); pUCD3960 (lane 3); pUCD3962 (lane 4), pUCD3964 (lane 5); pUCD3965 (lane 6). Purified Osa fusion protein (lane 2) as in lane 3 of Fig. 2. (**B**) Subcellular fractions: outer membrane (lane 1); inner membrane (lane 2); cytoplasm (lane 3); periplasm (lane 4). Lane 5: same as lane 2 of (**A**). The amount of protein in each lane was adjusted to represent that recoverable from a single *A. tumefaciens* cell. Numbers on the left are in kDa based on protein standards.

ing *osa* and either the penultimate locus orf3, or *nuc* and orf3, in clones pUCD3962 and pUCD3964, respectively, produced highly detectable Osa protein (Fig. 2). Each Osa-producing test strain containing pTiC58 was avirulent on Jimson weed (*Datura stramonium*) as anticipated (not shown). These results suggest that the *osa* operon is regulated in *A. tumefaciens*. This hypothesis is supported by the lack of expression of a promoter-less chloramphenicol acetyltransferase gene fused to *osa* of the *osa* operon in *A. tumefaciens* [3].

3.3. Osa is located at the inner-membrane

A. tumefaciens LBA4301(pTiC58Tra^c) cells containing pUCD3960 were separated into cytoplasmic, periplasmic, and inner- and outer-membrane fractions as described in Materials and methods. Proteins from each fraction were separated by SDS-PAGE and analysed by immunoblotting. As shown in Fig. 3, the Osa protein was found solely in the inner membrane fraction. The homogeneity of the inner and outer membrane preparations was verified by the presence of NADH oxidase activity (64 nmol min^{-1} (mg protein)$^{-1}$) only in the inner membrane fraction. The amount and subcellular location of this protein was not altered when *A. tumefaciens* cells were induced with acetosyringone, indicating that the regulation of *osa* is independent of acetosyringone induction. Western blot analysis of the subcellular fractions of *E. coli* DH5α containing pUCD3960 also showed that Osa is located at the inner membrane (not shown). These results indicate that Osa associates mainly with the inner membrane, irrespective of acetosyringone induction and bacterial chromosomal background.

3.4. Osa does not affect the production and subcellular location of VirB2, VirB3, VirB4, VirB9, and VirD4 proteins

Proteins encoded by *virB* and *virD* genes are essential for virulence of *A. tumefaciens*. Because Osa inhibits *A. tumefaciens* virulence completely, its mode of action may be directed at *vir* gene products. As we show here, the transcription and translation of the *vir* genes are not blocked, but neither protein-to-protein, nor competitive protein-to-membrane target interactions have been completely ruled out. Comparative analyses were therefore made of LBA4301(pTiC58Tra^c) cells containing either the *osa*-bearing plasmid pUCD3960, or the vector plasmid pUCD105 in the absence of *osa*. In either case, VirB2, VirB3, VirB4, VirB9 and VirD4 proteins were detected in the total membrane fraction (not shown). Upon fractionation of the membranes, Western blots revealed that VirB2 and VirB9 proteins were associated with both the inner and outer membranes as would normally be the case (Fig. 4), confirming previous VirB protein localization studies [11]. VirB4 and VirD4 were found in the inner membrane (not shown), as found previously in other studies [10,13,22]. These results show that in the presence of *osa*, the *virB* and *virD* operons are fully expressed.

In each instance, no differences were observed between the association of these Vir proteins with their respective membranes. Shifts in protein-to-membrane patterns can easily be detected as shown previously with the observed loss of membrane asso-

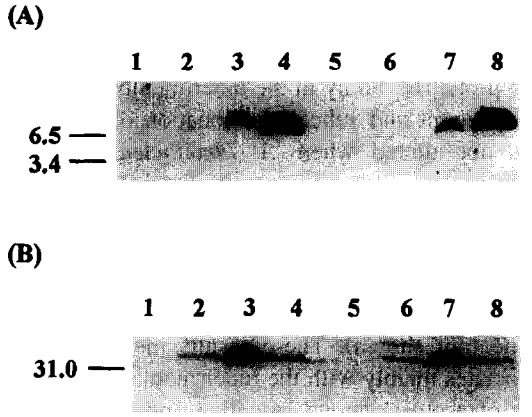

Fig. 4. Western blot analyses for the effect of Osa on the subcellular locations of VirB proteins in *A. tumefaciens*. Proteins were resolved by SDS-PAGE. (**A**) Proteins in each subcellular fraction were probed with VirB2 antibody: lanes 1 and 5 contain cytoplasmic proteins; lanes 2 and 6 contain the periplasmic fraction; lanes 3 and 7 contain the outer membrane proteins; and lanes 4 and 8 contain the inner membrane proteins. (**B**) The same sets of protein fractions were probed with VirB9 antibody. Proteins loaded in each lane were pre-adjusted to be equivalent on a per cell basis. The numbers on the left are positions of molecular mass standards in kDa.

ciation of VirB3 when VirB4 is mutated [23]. Hence, it appears that VirB2, VirB3, VirB9 and VirD4 remain unaffected by Osa in associating with their respective membranes.

4. Discussion

Osa protein produced in *E. coli* was isolated and purified, leading to the preparation of specific polyclonal antibody. This antibody was used to detect Osa in various subcellular fractions derived from *A. tumefaciens* cells containing pSa or a clone bearing the *osa* operon. These studies curiously showed the absence of the Osa protein in *A. tumefaciens* cells containing pSa or the entire *osa* operon, suggesting that the expression of *osa* may be tightly regulated and may require some type of external inducer. The nature of such an inducer is presently being explored in another study (L.-Y. Lee and C.I. Kado, unpublished). To circumvent the lack of *osa* expression in *A. tumefaciens*, transcription of the *osa* gene was placed under the control of a constitutive promoter derived from a kanamycin/spectinomycin operon [3,24]. Immunoblot analysis of the subcellular components of *A. tumefaciens* cells, containing the constitutively expressed *osa* gene, clearly showed the presence of Osa protein that specifically localizes to the inner membrane fraction. Examination of its amino acid sequence reveals a stretch of hydrophobic residues near the carboxyl terminus of Osa, which might serve to localize at least this part of the polypeptide to the membrane. The manner by which Osa spans the inner membrane awaits further studies.

The fact that Osa completely inhibits *A. tumefaciens* oncogenicity, and that the protein associates with the inner membrane as shown in this study, leads us to hypothesize that Osa might be blocking the proper generation of the postulated T-DNA transport apparatus which spans the membrane. Based on recent comparative amino acid sequence homology studies [5,11,22], and on the identification of a processed propilin-like VirB2 protein ([11]; A.L. Jones, E.-M. Lai and C.I. Kado, submitted), as well as electron microscopic observations ([10]; O. Chesnokova, J. Coutinho, I. Kahn, and C.I. Kado, submitted), the assembly of a conjugative pilin-like structure may be inhibited by Osa at its membrane location.

To assess this possibility, immunoblot studies using antibodies specific to various VirB proteins, including a VirD4 protein, were used to analyse *A. tumefaciens* cells harboring a Ti plasmid plus either an *osa* clone or the wild-type pSa plasmid. The results of these studies showed no obvious difference in the location or the amount of each VirB or VirD protein analysed in subcellular fractions of cells with and without *osa*. Thus, the Osa protein may either not be competing for specific sites on the membrane where VirB2, VirB3, VirB4, VirB9 and VirD4 proteins dock, or the interaction may be too subtle to detect at the sensitivity level of the immunoblot analyses, or the activity of Osa is not associated with these proteins. Although not all of the Vir proteins were analysed, the possibility that we have overlooked one that does react with Osa seems remote because recent cross-linking studies revealed no protein-to-protein interactions [26]. Additional studies underway using in situ electron microscopic analysis and immunogold-labelled antibodies should verify these results.

Previously, by comparative amino acid sequence homology studies, we found that there are relatively close homologies between the amino acid sequence of Osa and that of an IncP plasmid RP1 protein involved in fertility inhibition of IncW plasmids such as pSa [3]. Conjugative transfer requires the close association of specific proteins and plasmid DNA to form a complex at the membrane [24,25]. Fertility inhibition may result either through the alteration of such complexes by a protein identified to inhibit plasmid conjugative transfer, or by plugging the postulated pore through which the donor DNA must pass into the recipient cell. Thus, one of these mechanisms is that used by Osa. Our recent studies using agroinfection and intron-β-glucuronidase (GUS) assays to measure the transfer of the T-DNA from *A. tumefaciens* to plants indicate that T-DNA transfer is indeed inhibited by Osa [26]. This further supports the notion that Osa may be interfering with the T-DNA transfer mechanism.

Acknowledgements

We thank Louise Jones and Ken Shirasu for technical assistance and helpful discussions. Special thanks goes to Shih-Tung Liu for generously providing laboratory space and equipment at Chang-Gung Medical College, Taoyan, Taiwan during a summer stay by the senior author. This work was supported by US Public Health Service grant GM45550 from National Institutes of Health.

References

[1] Loper, J.E. and Kado, C.I. (1979) Host range conferred by the virulence-specifying plasmid of *Agrobacterium tumefaciens*. J. Bacteriol. 139, 591–596.

[2] Close, S.M. and Kado, C.I. (1991) The *osa* gene of pSa encodes a 21.1-kilodalton protein that suppresses *Agrobacterium tumefaciens* oncogenicity. J. Bacteriol. 173, 5449–5456.

[3] Chen, C.-Y. and Kado, C.I. (1994) Inhibition of *Agrobacterium tumefaciens* oncogenicity by the *osa* gene of pSa. J. Bacteriol. 176, 5697–5703.

[4] Close, S.M. and Kado, C.I. (1992) A gene near the plasmid pSa origin of replication encodes a nuclease. Mol. Microbiol. 6, 521–527.

[5] Kado, C.I. (1994) Promiscuous DNA transfer system of *Agrobacterium tumefaciens*: role of the *virB* operon in sex pilus assembly and synthesis. Mol. Microbiol. 12, 17–22.

[6] Kado, C.I. (1994) T-DNA transfer to plants is mediated by pilus-like apparatus encoded by the Ti plasmid *virB* operon. In: Advances in Plant Biotechnology (Ryu, D.D.Y. and Furusaki, S. Eds.), pp. 23–36. Elsevier, Amsterdam.

[7] Close, S.M. (1991) Ph.D. thesis. University of California, Davis, CA.

[8] Tait, R.C., Rempel, H., Rodriguez, R.L. and Kado, C.I. (1985) The aminoglycoside-resistance operon of the plasmid pSa: nucleotide sequence of the streptomycin–spectinomycin resistance gene. Gene 36, 97–104.

[9] Ward, J.E. Jr., Dale, E.M., Nester, E.W. and Binns, A.N. (1990) Identification of a VirB10 protein aggregate in the inner membrane of *Agrobacterium tumefaciens*. J. Bacteriol. 172, 5200–5210.

[10] Berger, B.R. and Christie, P.J. (1993) The *Agrobacterium tumefaciens virB4* gene product is an essential virulence protein requiring an intact nucleoside triphosphate-binding domain. J. Bacteriol. 175, 1723–1734.

[11] Shirasu, K. and Kado, C.I. (1993) Membrane location of the Ti plasmid VirB proteins involved in the biosynthesis of a pilin-like conjugative structure of *Agrobacterium tumefaciens*. FEMS Microbiol. Lett. 111, 287–294.

[12] Beijersbergen, A., Smith, S.J. and Hooykaas, P.J.J. (1994) Localization and topology of VirB proteins of *Agrobacterium tumefaciens*. Plasmid 32, 212–218.

[13] Thorstenson, Y.R., Kuldau, G.A. and Zambryski, P.C. (1993) Subcellular localization of seven VirB proteins of *Agrobacterium tumefaciens*: implications for the formation of a T-DNA transport structure. J. Bacteriol. 175, 5233–5241.

[14] Thorstenson, Y.R. and Zambryski, P.C. (1994) The essential virulence protein VirB8 localizes to the inner membrane of *Agrobacterium tumefaciens*. J. Bacteriol. 176, 1711–1717.

[15] Studier, F.W. and Moffatt, B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selection of high level expression of cloned genes. J. Mol. Biol. 189, 113–130.

[16] Rosenberg, A.G., Lade, B.N., Chiu, D., Dunn, J.J. and Studier, W. (1987) Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene 56, 125–135.

[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. 2 edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

[18] Cangelosi, G.A., Ankenbauer, R.G. and Nester, E.W. (1990) Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proc. Natl. Acad. Sci. USA 87, 6708–6712.

[19] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

[20] Valentine, C.R.I. and Kado, C.I. (1989) Molecular genetics of IncW plasmids. In: Promiscuous Plasmids of Gram-negative Bacteria (Thomas, C.M., Ed.), pp. 125–163. Academic Press, London.

[21] DeMaagd, R.A. and Lugtenberg, B. (1986) Fractionation of

Rhizobium leguminosarum cells into outer membrane, cytoplasmic membrane, periplasmic and cytoplasmic components. J. Bacteriol. 167, 1083–1085.

[22] Shirasu, K., Koukolíková-Nicola, Z., Hohn, B. and Kado, C.I. (1994) An inner-membrane-associated virulence protein essential for T-DNA transfer from *Agrobacterium tumefaciens* to plants exhibits ATPase activity and similarities to conjugative transfer genes. Mol. Microbiol. 11, 581–588.

[23] Jones, A.L., Shirasu, K. and Kado, C.I. (1994) The product of the *virB4* gene of *Agrobacterium tumefaciens* promotes accumulation of VirB3 protein. J. Bacteriol. 176, 5255–5261.

[24] Frost, L.S., Ippen-Ihler, K. and Skurray, R.A. (1994) Analysis of the sequence and gene products of the transfer region of the F sex factor. Microbiol. Rev. 58, 162–210.

[25] Willetts, N. and Skurray, R. (1987) Structure and function of the F factor and mechanism of conjugation. In: *Escherichia coli* and *Salmonella typhimurium*, Cellular and Molecular Biology (Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M., Umbarger, H.E., Eds.), pp. 1110–1133. American Society for Microbiology, Washington, D.C.

[26] Lee, L.-Y. and Kado, C.I. (1995) The oncogenic suppressive gene *osa* is part of an operon in plasmid pSa and encodes a 21 kDa protein which blocks T-DNA conjugative transfer from *Agrobacterium* to plants. Proc. Int. Conf. *Agrobacterium* and Beyond, Abstr. Gif-sur-Yvette, September 1995.